2,6-Diaminopimelic acid as a biological marker of rumen synthesis and fermentation capacities in the transition period and early lactation of dairy cows

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Abstract

The experiment was conducted to evaluate the effect of different types of available nutrients of total mix rations on fermentation and synthesis capacities of the rumen in Holstein dairy cows divided into groups in the transition period ($\pm$ 3 weeks after parturition) and early lactation (60–80 days in milk). The diets were fed as a total mixed ration. Results obtained with feeding Diet C (high concentration of available nutrients) to dairy cows confirmed a significant increase in 2,6-diaminopimelic acid from the initial mean 2.2 μg/ml at 21 days before calving to 6.2 μg/ml ($P < 0.01$) at 21 days after calving or 7.6 μg/ml ($P < 0.01$) at early lactation, and a parallel significant increase in the concentration of volatile fatty acids ($P < 0.01$). The concentration of propionic acid increased from the initial mean 25.3 to 39.3 mmol/l ($P < 0.01$) in the transition period, and to 43.1 mmol/l at early lactation ($P < 0.01$). All indicators of rumen fermentation and synthesis determined in dairy cows with Diet B (low concentration of available nutrients) of the total mix ration according to productive phases were not significant. High regression coefficients of examined indicators of 2,6-diaminopimelic acid to volatile fatty acid (r = 0.813), propionic acid (r = 0.780) and acetic acid (r = 0.635) indicate direct correlation between fermentation and synthesis activities of the rumen. Our results show that 2,6-diaminopimelic acid appears as a perspective marker for the monitoring and evaluation of the synthesis function of the rumen and production of bacterial biomass under breeding conditions.

VFA, DAPA, feeding and nutrition, microflora, fermentation

Rumen fermentation of the feed's organic matter and microbial biomass synthesis are reported to have the potential to meet 70–85% of the energy requirements and 70–100% of the protein needs of ruminants, even at higher levels of production. Microbial proteins synthesized in the rumen cover 80% of daily amino acid needs and constitute 50–80% of total absorbable amino acids (Bach et al. 2005). Synthesis of microbial proteins is affected by usability of fermentable carbohydrates (Ahvenjarvi et al. 2002), nitrogen (Verbic 2002) and minerals (Broudiscou et al. 1999). Optimum production of rumen microflora and synthesis of microbial proteins at the level of 12.6 g/100 g fermentable organic matter is ensured when the concentration of NH_3 in the ruminal fluid varies in the range 8.8–14.7 mmol/l which is an accepted reference value for assessment of the level of protein saturation in ruminant rations (Vajda et al. 1994; NRC 2001). Microbial efficiency expressed as g of microbial nitrogen per kg of dry matter, organic matter, total carbohydrate, and 100 g N digested, ranged from 20.8 to 26.6; 34.7 to 44.3; 35.1 to 50.3 and 89 to 95 g, respectively (Sniffen et al. 2006). Multiplication and growth of rumen microflora is affected by nutritional (quantity and ratio of carbohydrates and proteins) and dietetic factors (structure of total mix ration, dry matter intake, production of saliva). These factors are directly related to rumen pH, intensity and type of fermentation, osmolality, passage and evacuation of solids and liquids (Dehority 2004). Quantification of synthesis of microbial proteins is an important part of the evaluation of biological control of the nutritional level of high yielding dairy cows. Several methods have been developed for

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the determination of microbial protein in the rumen contents. These methods fall into two categories: methods for tracing bacterial protein or those based on the analysis of marker components believed to be uniquely microbial in origin. In particural, 2,6-diaminopimelic acid (DAPA) was accepted as a reliable marker for determining microbial protein (Miner-Williams et al. 2013). It is a well known peptidoglycan amino acid bound to bacterial cell walls of gram-negative bacteria (Ling 1990). Gram-positive bacteria contain approximately 30–60% peptidoglycan, whereas gram-negative bacteria have only 10% peptidoglycan (Schoenhusen et al. 2008). Amino acid composition and structure of peptidoglycans in gram-positive bacteria varies greatly, whereas that of gram-negative bacteria is more consistent (Miner-Williams et al. 2013). Knowledge of the DAPA concentration in the walls of bacterial cells is the basis for verification of the hypothesis of the possibility of applying DAPA in the rumen content as a marker of the synthesis of bacterial biomass.

The aim of the study was to investigate the influence of ration composition on the level and dynamics of rumen fermentation of carbohydrates and degradation of proteins in relation to the synthesis of microbial proteins by observing 2,6-diaminopimelic acid.

Materials and Methods

The feeding experiment was carried out as two consecutive experiments on dairy cows with diets varying in the sources of rumen available carbohydrate and protein, to analyze the influence of the nutrient level on fermentation and synthesis capacity of the rumen. Diets were formulated using the NRC (2001). Eighteen multiparous Holstein dairy cows (600 ± 12.5 kg body weight (BW); 2.9 ± 0.3 parity) were studied during three periods: 1) from day 21 before expected calving; 2) from calving up to day 21 of lactation; 3) day 60 to day 80

Table 1. Ingredients and chemical composition of TMR - Diet C.

		3 week before calving	3 weeks after calving	60–80 DIM
Corn silage	kg	5.0	9.0	9.0
Alfalfa haylage	kg	6.0	8.0	8.0
Grass hay	kg	1.5	-	-
Alfalfa hay	kg	-	3.0	3.0
Barley silage	kg	5.0	9.0	9.0
Barley straw	kg	0.5	-	-
Grain mixture	kg	3.0	7.0	7.0
Corn grain	kg	2.0	5.0	5.0
Amygold	kg	1.0	3.0	3.0
DM	%	39.4	51.1	56.2
CP	g/kg of DM	131.9	153.9	170.3
NDF	g/kg of DM	471.1	359.6	309.1
NFC	g/kg of DM	314.5	381.7	405.2
Starch	g/kg of DM	236.7	272.7	279.7
NEL	MJ/kg of DM	6.24	6.98	7.31
Daily intake of nutrients				
DM	kg	11.7	17.0	23.6
CP	g	1543.2	2614.6	4019.1
NDF	g	5511.9	6113.2	7294.8
NFC	g	3679.7	6488.9	9562.7
Starch	g	2769.4	4635.9	7011.6
NEL	MJ	73.01	118.7	172.5

TMR – total mixed ration, DM – dry matter, CP – crude protein, NDF – neutral detergent fibre, NFC – non-fibre carbohydrates, NEL – net energy for lactation, MJ – mega joule, DIM – days in milk, Amygold – corn gluten feed (Beuker)

Table 2. Ingredients and chemical composition of TMR - Diet B.

		3 week before calving	3 weeks after calving	60-80 DIM
Grass silage	kg	10.0	15.0	15.0
Corn silage	kg	5.0	15.0	15.0
Hay	kg	1.0	1.0	1.0
Rindavit[2]	kg	0.2	-	-
Grain mixture	kg	3.0	7.0	7.0
DM	%	53.2	41.9	43.1
CP	g/kg of DM	97.5	149.1	140.2
NDF	g/kg of DM	570.3	426.1	432.8
NFC	g/kg of DM	191.1	311.1	312.4
Starch	g/kg of DM	180.9	257.1	262.5
NEL	MJ/kg of DM	5.40	6.37	6.34
Daily intake of nutrients				
DM	kg	11.9	15.0	17.0
CP	g	1160.3	2236.5	2453.5
NDF	g	6785.6	6391.5	7574.0
NFC	g	2274.1	4666.5	5467.0
Starch	g	2152.7	3856.5	4593.7
NEL	MJ	64.3	95.5	110.9

TMR – total mixed ration, DM – dry matter, CP – crude protein, NDF – neutral detergent fibre, NFC – non-fibre carbohydrates, NEL – net energy for lactation, MJ – mega joule, DIM – days in milk, Rindavit - the vitamin/mineral premix (Schaumann)

in milk. Cows were housed in box stalls and milked in these stalls at 06:00 h and 18:00 h. Diets were fed as a total mixed ration (TMR) *ad libitum* at 07:00 h and 19:00 h. The ingredient and chemical composition of the diets and daily intake of nutrients are presented in Tables 1 and 2. The TMR for different groups of cows was calculated to meet the demands for energy and protein of the cow (600 kg of BW) producing 21 kg of milk/d with an assumed dry matter intake (DMI) of 16 kg of dry matter/day (DM/d) in the second group, and 32 kg of milk/d and 21 kg DMI in the third group. The diets varied in sources of rumen available carbohydrate and protein (Diet C – high concentration of available nutrients, Diet B – low concentration of available nutrients). Samples of TMR were analyzed for dry mater, crude protein (CP), acid and neutral detergent fibre (ADF, NDF), and starch according to conventional methods (Committee Regulation EC No.152/2009 of January 27, 2009). Dry matter was determined by weight upon drying the sample at 105 °C under prescribed conditions. The CP content was determined according to the Kjeldahl methods ($N \times 6.25$) using a 2300 Kjeltec Analyser Unit (Foss Tecator AB, Hoganas, Sweden). The ADF and NDF were determined by the device Dosi-Fibre Analyzer (JP SELECTA, Spain) and non-fibre carbohydrates (NFC) were calculated by difference (100− (CP + (NDF–NDF bound protein) + ash + ether extract)).

Analysis of VFA and NH_3

Samples of the rumen contents intended for the analysis of fermentation and synthesis capacity of the rumen were taken 4–5 h after morning feeding by stomach cannulas. Samples of rumen fluid were strained through 3-layer of gauze. VFA in the rumen contents were determined in a two-capillary isotachophoretic analyser EA100 (VILLA LABECO, Slovak Republic). The pH of the rumen contents was determined potentiometrically with portable electronic pH-meter (JP SELECTA, Spain). The concentration of NH_3 in samples of rumen contents was determined by a Kjeldahl-N using a 2300 Kjeltec Analyser Unit (Foss Tecator AB, Hoganas, Sweden).

Analysis of 2,6-diaminopimelic acid (DAPA)

After acidic hydrolysis of the sample of ruminal contents, DAPA was determined by means of AAA 400 (INGOS, Czech Republic). Immediately after sampling, the samples were conserved with addition of thymol. They were stored in a portable refrigerator and transported to the laboratory for examination within 1 h. Before processing, the samples were refrigerated for 1 h and filtered through 3-layer gauze. Then a 10 ml aliquot of rumen contents was pipetted into a hydrolysation flask and 50 ml of 6 N HCl were added. Hydrolysis was carried out for 23 h in a thermostat at 110–112 °C. After 23 h, the hydrolysation reagent was evaporated in a vacuum

rotating evaporator using an oil vacuum pump. The content of DAPA was determined by the method of ion exchange chromatography with post-column derivatisation with ninhydrine, using an analyser of amino acid AAA 400 (INGOS, Czech Republic).

The results achieved were processed by mathematical-statistical methods involving the statistical program GraphPad Prism4. We evaluated values and their differences using Tukey-HSD test at significance levels of $P \leq 0.01$ and $P \leq 0.05$, respectively. Each indicator was presented by its mean (x) and standard deviation (S.D.).

Results

Diet C (high concentration of available nutrients)

In the group of dairy cows in the period of nutritional adaptation for parturition, analyses showed that the content of all nutrients in TMR corresponded to the recommended values with the exception of increased NDF. The intake of nutrients corresponded to the profile of rumen fermentation of carbohydrates (Table 3) with mean values of pH 6.4 ± 0.1 and VFA 110.1 ± 5.4 mmol/l. The proportions of acetic acid (C_2) and propionic acid (C_3) reached $66.1 \pm 2.9\%$ and $22.9 \pm 3.2\%$, respectively, and their ratio was 2.91 : 1 ($C_2 : C_3$) which reflected a high degree of adaptation of rumen fermentation in the phase of preparation for the intake of the concentrate type of rations after parturition. The intake and rumen degradation of CP maintained the concentration of NH_3 in the rumen close to the lower reference limit and the content of DAPA at 2.2 ± 0.3 μg/ml.

Table 3. Indicators of rumen fermentation and 2,6-diaminopimelic acid in rumen content of dairy cows.

Indicator		1–3 weeks *pre partum*	1–3 weeks *post partum*	60–80 DIM *post partum*	Summary
DAPA μg/ml	C	$2.2 \pm 0.3^{a,b}$	6.2 ± 1.1^a	7.6 ± 2.7^b	5.3 ± 2.9
	B	1.8 ± 0.5	1.9 ± 0.4	1.9 ± 0.8	1.9 ± 0.6
pH	C	6.4 ± 0.1^i	6.1 ± 0.3^h	$5.6 \pm 0.2^{h,i}$	6.0 ± 0.4
	B	6.9 ± 0.2	6.8 ± 0.1	6.96 ± 0.4	6.9 ± 0.3
NH_3 mmol/l	C	$10.5 \pm 2.3^{j,l}$	$15.2 \pm 2.7^{j,k}$	$21.5 \pm 3.6^{k,l}$	15.7 ± 5.3
	B	3.5 ± 0.6	8.6 ± 2.1	8.6 ± 1.6	6.9 ± 2.9
VFA mmol/l	C	$110.1 \pm 5.4^{c,e}$	$127.7 \pm 8.7^{c,d}$	$148.4 \pm 12.6^{d,e}$	128.7 ± 18.3
	B	86.5 ± 14.3	106 ± 6.2	87.9 ± 22.9	93.5 ± 18.3
Acetic acid %	C	66.1 ± 2.9	56.3 ± 3.3	58.2 ± 3.4	60.2 ± 5.3
	B	70.5 ± 2.0	67.7 ± 1.3	68.2 ± 2.7	68.8 ± 2.4
Propionic acid %	C	22.9 ± 3.2	30.7 ± 2.8	29.0 ± 1.6	27.5 ± 4.2
	B	18.9 ± 2.4	20.8 ± 1.3	19.8 ± 2.5	19.9 ± 2.3
$C_2{:}C_3$	C	2.91 : 1	1.9 : 1	2.02 : 1	2.28 : 1
	B	3.8 : 1	3.3 : 1	3.52 : 1	3.52 : 1

C – Diet C (high concentration of available nutrients), B – Diet B (low concentration of available nutrients), DAPA – 2,6-diaminopimelic acid, VFA – volatile fatty acid, C2 –acetic acid, C3 – propionic acid, DIM – days in milk, a,e,g,i,l: $P < 0.001$, b.c.f: $P < 0.01$, d,h,k,j: $P < 0.05$

Group of *post partum* dairy cows

In the puerperal stage up to 21 days postpartum, the obtained results indicated a high degree of rumen fermentation and optimum adaptation to the diet C (resp. concentrate type rations). The high concentration of VFA reaching 127.7 ± 8.7 mmol/l at marginal levels of rumen pH 6.1 ± 0.3, decreased proportion of C_2 $56.3 \pm 3.3\%$, and increased level of C_3 $30.7 \pm 2.8\%$ resulting in their decreased ratio of 1.9:1 ($C_2{:}C_3$), confirmed appropriate fermentation of the concentrate type TMR. The concentrations of NH_3 determined in the

rumen contents (15.2 ± 2.7 mmol/l) were close to the upper reference limit and correlated with the proportion of proteins in TMR, which indicated saturation of needs for CP required for multiplication of rumen microflora, and the concentration of DAPA (6.2 ± 1.1 µg/ml) showed a highly significant ($P < 0.01$) increase.

Group of dairy cows at early lactation

These animals were fed the concentrate type of TMR with a 40% content of NFC and 30% content of starch, and showed very high level of rumen fermentation. The mean values of VFA reaching 148.4 ± 12.6 mmol/l, rumen pH at the level of 5.6 ± 0.2, proportions of C_2 and C_3 at 58.2 ± 3.4% and 29.0 ± 1.6%, respectively, and their mutual ratio of ($C_2 : C_3$) 2.02 : 1 signalled chronic rumen acidosis while maintaining rumen fermentation capacity. At early lactation, in addition to increased synthesis capacity of the rumen expressed as DAPA reaching 7.6 ± 2.7 µg/ml we were able to observe an increased NH_3 concentration of 21.5 ± 3.6 mmol/l ($P < 0.01$) signalling secondary degradation of microbial proteins conditional on a decrease of pH and deterioration of conditions for the survival of rumen microflora. The results of examination of rumen metabolism in relation to the nutrient intake according to productive stages proved a significant increase in DAPA from the mean initial *pre partum* concentration of 2.2 µg/ml to 6.2 µg/ml ($P < 0.01$) at 21 days *post partum* and 7.6 µg/ml ($P < 0.01$) at early lactation in parallel with a significant increase in VFA from the *pre partum* concentration of 110.1 mmol/l to 127.7 mmol/l *post partum* ($P < 0.01$) and 148.4 mmol/l at early lactation ($P < 0.0001$). The concentration of propionic acid increased from the *pre partum* level of 25.3 mmol/l to 39.3 mmol/l ($P = 0.0022$) *post partum* and to 43.1 mmol/l ($P < 0.01$) at early lactation.

Diet B (low concentration of available nutrients)

Low intake of energy (5.4 MJ/kg) and of CP equal to 9.7% in TMR dry matter in the phase of preparation for parturition affected essentially the level of rumen fermentation. High level of pH 6.9 ± 0.2, limited production of VFA (86.5 ± 14.3 mmol/l), increased proportion of acetic acid 70.5 ± 2.0% and reduction in propionic acid to 18.9 ± 2.4 % with high ratio of C2: C3 equal to 3.8: 1 confirmed that rumen fermentation corresponded to Diet B with the mean values of DAPA at 1.8 ± 0.5 µg/ml. In the *post partum* stage and at early lactation the dairy cows were fed unified rations whose composition during the productive phase (CP 14–15%, NEL 6.3 MJ and PDI 88 g/kg) corresponded to Diet B (low concentration of available nutrients, or bulk type of TMR). The indicators of rumen fermentation in the *post partum* phase and at early lactation indicated that degradation of carbohydrates (VFA, pH, $C_2:C_3$), concentration of NH_3, and synthesis capacity of rumen microflora (DAPA) corresponded to a low nutrient intake reflecting the degree of fermentation (Table 3). All the indicators of fermentation and rumen synthesis analyzed with TMR of Diet B, according to production phases, showed insignificant differences.

Relationship between fermentation and synthesis capacities of rumen microflora

Regression relationships of the investigated concentrations of DAPA, VFA, propionic and acetic acids, and their ratios were obtained by mutual comparison of results determined for concentrate and bulk type rations according to production phases. Evaluation of the relationship between VFA and DAPA proved a direct regression relationship with high regression coefficient (r = 0.813). In relation to the nutrient content of the analyzed rations the correlation confirmed existence of a direct relationship between fermentation activity - production of VFA and the synthesis activity and development of rumen microflora with DAPA as its direct cellular marker. When analyzing the relationship between individual VFA and the level of DAPA in the rumen contents of dairy cows, the highest correlation was found

for propionic acid with a linear regression r = 0.780, and for both acetic acid and DAPA with a regression coefficient r = 0.635. The $C_2 : C_3$ ratio calculated from the concentrations of the respective volatile fatty acids allowed us to characterize the fermentation model in relation to DAPA in the rumen contents and to obtain the regression coefficient r = 0.734.

Discussion

The time of sample collection has no effect on the content of DAPA in bacteria, and the ratio of DAPA-N concentration to total N concentration in mixed rumen bacteria after feeding remains relatively constant (Dufva et al. 1982). Our observations of the relationship between markers of the fermentation (VFA, pH) and synthesis activities of the rumen (DAPA) were therefore carried out at the time of maximum rumen fermentation of carbohydrates and degradation of proteins, and samples of ruminal fluid were taken 4–6 h after feeding. The present model feeding experiment investigated relationships between the fermentation and synthesis capacities for bulky and concentrate types of rations. The determined concentrations of DAPA, together with the levels of VFA, pH and rumen NH_3, corresponded to marginally increased levels with tendency to acidification.

Rumen synthesis of microbial proteins depends considerably on the quantity and type of ingested carbohydrates contained in the ration as a source of energy for the synthesis of peptide bonds and growth of micro-organisms (Sinclair et al. 1995). Olubobokun et al. (1988) observed that the concentration of DAPA in the ruminal fluid was higher compared to rumen microflora which adheres to feed particles. A portion of DAPA from the rumen liquid fraction is not precipitated even by centrifugation at higher speed which proves that it occurs in the form of very small particles as a part of fragments of cellular walls produced at microbial lyses and degradation of bacterial proteins.

Bacterial lyses of rapidly growing population which increase the concentration of DAPA result in overrating the synthesis capacity of microbial proteins (Gomez et al. 1991). Our observations indicated such a state of rumen metabolism in groups of animals with tendency towards rumen acidification in which we observed also high concentrations of NH_3 and DAPA. Feeding the concentrate type of rations with a higher content of VFA, low rumen pH and altered ratio of propionic and acetic acids reflects higher quantity of substrates from fermentation which supports dominance of amylolytic population and suppresses celulolytic population. Higher proteolytic activity is typical for amylolytic microflora compared to celulolytic strains (Wallace 1987) which explains higher degradation of proteins with an increasing concentration of DAPA. Low rumen pH, approaching the limit of subclinical acidosis, supports multiplication of *Streptococcus bovis*.

It was assumed that *S. bovis* does not contain DAPA (Dufva et al. 1982). Later analyses proved the presence of DAPA in *S. bovis* (Russell and Robinson 1984) which at concentrate feed rations and low pH may increase the rumen content of DAPA in dairy cows. The growth phase of microflora is the time of incubation and differs from availability of substrates and type of microbial strains that are manifested by varying microbial synthesis (Blümmel et al. 2003). Because of those different concentrations it may reflect various types of micro-organisms within the rumen population, different ratios of protozoa and bacteria, bacteria in liquid and solid phases of the rumen contents and also various stages of growth of respective microbial populations. The diverse bacterial populations within rumen microflora are characterized by different cellular walls and peptidoglycan layers which are reflected in varying concentrations of DAPA in the rumen contents (El-Waziry et al. 2005).

Increasing proportion of the concentrate feed in rations increases the abundance of gram positive bacteria with relatively thick peptidoglycan layer. The DAPA-N : total bacterial N ratio differs within bacterial species in dependence on the type of rations (Dufva et al.

1982). The natural variability of DAPA proportion in individual microbial strains limited the wider use of DAPA as a biological marker of growth of microbial population. The 2,6-diaminopimelic acid appears as a perspective marker for monitoring and evaluation of the synthesis function of the rumen and production of bacterial biomass under breeding conditions.

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